ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 8 (2): 276-280, 2009
ISSN 1682-296X

Application of Protease Isolated from *Bacillus* sp. 158 in Enzymatic Cleansing of Contact Lenses

[1]Rasika Pawar, [2]Vasudeo Zambare, [1]Siddhivinayak Barve and [1]Govind Paratkar
[1]Department of Biotechnology, Vinayak Ganesh Vaze College, Mulund (E), Mumbai 400081, India
[2]Department of R and D, Advanced Enzyme Technologies Ltd., Nasik 422113, India

Abstract: A neutral protease, isolated from *Bacillus* sp. 158 was used for removing protein deposits from contact lenses. Partial purification of the protease was carried out using ammonium sulphate and factors affecting the enzyme activity, such as assay temperature and assay pH were characterized. The optimum pH and temperature for protease were found to be pH 7.0 and 30°C, respectively. The partially purified protease was stable at temperature range of 30-40°C and pH 6-7. However, protease was maximum stable at 30°C and pH 7.0. The enzyme could be effectively used to remove protein deposit from contact lenses indicating its potential to increase in transmittance of lenses.

Key words: Neutral protease, *Bacillus* sp. 158, contact lenses cleansing

INTRODUCTION

Protease constitutes one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (Nunes and Martins, 2001). Proteases are of commercial value and find multiple applications in various industrial sectors. Proteases are widely used in detergent, food and leather tanning industries (Abidi *et al*., 2008; Zambare *et al*., 2007; Kumar and Takagi, 1999). Several alkaline proteases were reported for hydrolysis of fibrous proteins of horn, feather and hair and their application for various value added byproducts (Anvar and Saleemuddin, 1998; Giongo *et al*., 2007). Other potential industrial applications of alkaline protease include the utilization in peptide synthesis, in the resolution of the racemic mixture of amino acids, in the hydrolysis of gelatin layers of X-ray films and in the recovery of silver (George *et al*., 1995; Gupta *et al*., 2002; Singh *et al*., 1999).

In normal course of wearing contact lenses, tear films and proteinaceous debris have a tendency to deposit up on lens surfaces, which affect the optical clarity of the lenses. Also, contact lens surface deposits increase the potential of many pathogens including adhesion of *Pseudomonas aeruginosa* (Butrus and Klotz, 1990; Bruinsma *et al*., 2001). The debris deposited on contact lenses during their ophthalmic use, mainly consist of proteins. Mainly, contact lens cleansing solutions have been prepared using plant (papain) and animal (pancreatin, trypsin and chymotrypsin) proteases. Several microbial enzymes from *Bacillus* sp., *Streptomyces* sp. and *Aspergillus* sp. were reported for cleansing of tear films and debris of contact lens. However, in most instances they impart an unpleasant odor to the cleansing bath or develop an odor after a few hours of use. With the view of overcoming these drawbacks and to make the cleansing composition odorless and safe i.e., not producing an allergic response or causing irritation to the eyes, bacterial proteases are gaining importance. Several reports are available on production of proteases from bacterial cultures and *Bacillus* sp. is the dominating organism (Joo and Chang, 2005; Tari *et al*., 2006; Nilegaonkar *et al*., 2007). Therefore, it is essential to explore bacterial protease based cleansing solutions for lens cleansing.

The present study describes the production and properties of protease from *Bacillus* sp. 158 and its application in contact lens cleansing.

MATERIALS AND METHODS

Microorganism and enzyme production: The bacterium used in this study was *Bacillus* sp. 158, a fish waste isolate. The stock culture was maintained in 15% glycerol at -20°C. By inoculating a loopful of culture in Nutrient broth primary inoculum was developed. The productivity medium 50 mL (1.0% glucose, 0.5% yeast extract, 0.5% peptone, 0.2% KH_2PO_4, 1.0% Na_2CO_3, pH 7.0) was inoculated with 5% of 24 h old (10^7 cells mL^{-1}) and incubated at 30°C for 24 h under shake culture condition (150 rpm). The broth was centrifuged at 10,000 g for 10 min to obtain Cell Free Supernatant (CFS). The CFS was then

Corresponding Author: Dr. Vasudeo Zambare, Department of R and D, Advanced Enzyme Technologies Ltd., Nasik 422113, India

partially purified by 40% saturation of ammonium sulphate. The precipitated enzyme was suspended in phosphate buffer (pH 7.0) and used for further study.

Protease assay: Protease activity was measured using caseinolytic assay (Zambare *et al*., 2007) with some modifications. The culture supernatant (0.1 mL) was incubated in 9 mL of 1% casein at 30°C for 20 min. The reaction was stopped by 1.5 mL of trichloroacetic acid (5%) and the casein hydrolysis product was measured by modified Folin Ciocalteu method (Lowry *et al*., 1951), against inactive enzyme. A standard graph was generated using standard tyrosine of 10-50 μg mL^{-1}. One unit of protease activity was defined as the amount of enzyme, which liberated 1 μg tyrosine per min at 30°C.

pH and temperature activity and stability: The effect of pH on the enzyme activity was determined by incubating the partially purified protease between pH 4.0 and 9.0 using buffers of different pH (0.1 M acetate buffer, pH 4-6 and 0.1 M Tris-HCl buffer, pH 7-9). The effect of temperature on the enzyme activity was determined by incubating the partially purified protease at different temperatures ranging from 20-60°C with casein as substrate. The pH stability of partially purified protease was determined with casein (1% w/v) as a substrate dissolved in different buffers. The pH stability of the protease was determined by preincubating the enzyme in different buffers (6-8) up to 90 min at 30°C. Likewise, thermal stability of the precipitated protease was determined by pre-incubating the enzyme at different temperatures from 30-50°C up to 90 min in buffer of pH 7. All experiments were carried out using the standard assay condition in duplicate and each analysis was also performed in duplicate.

Enzymatic lens cleansing: A filter sterilized artificial tear solution was prepared with 0.2% lysozyme in electrolyte solution (0.22 g Na_2CO_3 and 0.7 g NaCl, pH 7.5). This solution was heated at 50°C for 20 min to denature lysozyme protein and used for contact lens coating. Before initiating the coating and cleansing process, light transmission reading for all soft contact lenses (Bausch and Lomb, Rochester, NY) used in the study was recorded using spectroscope (Konica Minolta CM 3500d) at 500 nm.

Contact lenses were placed in 2 cm diameter sterile petri dish and soaked in the 3 mL filter sterilized artificial tear solution for 20 min at 30°C to coat the lens with lysozyme protein and light transmission readings were recorded. Lenses employed for enzyme treatment were then transferred to 3 mL crude enzyme (30 U mL^{-1}) and placed in 2 cm sterile petri dish. Enzyme treatment was done for 30, 60 and 90 min at 30°C. Light transmission readings were recorded post enzyme treatment. Similarly, a control set of lenses was soaked in tear solution but treated with phosphate buffer (pH 7.0) instead of enzyme and light transmission readings were recorded in similar way as mentioned above. Protein removal was spectrophotometrically assayed in visible range according to method described by Harris *et al.* (2000) with some modifications.

Data analysis: All data used for this experimentation is obtained from duplicate experiments. Statistical analysis was done by using Student's t-test

RESULTS AND DISCUSSION

A bacterial culture of *Bacillus* sp. 158, a fish waste isolate was identified at genus level by comparing the test results with Bergey's Manual of Systematic Bacteriology (Sneath *et al*., 2005). Several *Bacillus* sp. produced variety of proteases and has major application in detergent industry (Anwar and Saleemuddin, 1998).

Isolated protease from *Bacillus* sp. 158, showed activity of 30 and 500 U mL^{-1} in crude CFS and precipitated enzyme with protein content of 200 and 600 mg mL^{-1}, respectively. Haq *et al.* (2003) reported the maximum protease activity during the course of study was 4.8 U mL^{-1}.

The enzyme was active in the pH range of 5-9, with optimum activity at pH 7 (Fig. 1) suggesting presence of neutral protease. However, 90% of activity was still retained at pH 8 and 80% at pH 6. Likewise, Sidler *et al.* (1986) reported optimum pH of 6.8 for *B. cereus* protease. The preliminary studies on the extracellular protease secreted by the *Bacillus* sp. showed that it has dual pH maxima, at 7.5 and 9 (Annapurna *et al*., 1996).

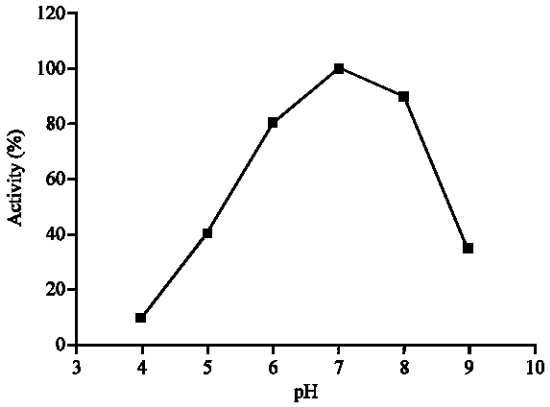

Fig. 1: Effect of pH on enzyme protease activity of protease produced by *Bacillus* sp. 158. (100% activity corresponds to 30 U mL^{-1})

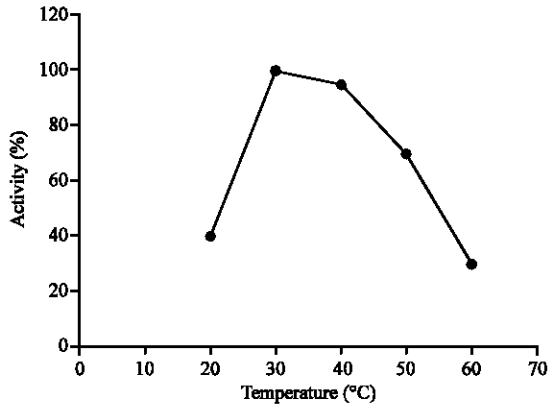

Fig. 2: Effect of temperature on enzyme protease activity of protease produced by *Bacillus* sp. 158. (100% activity corresponds to 30 U mL^{-1})

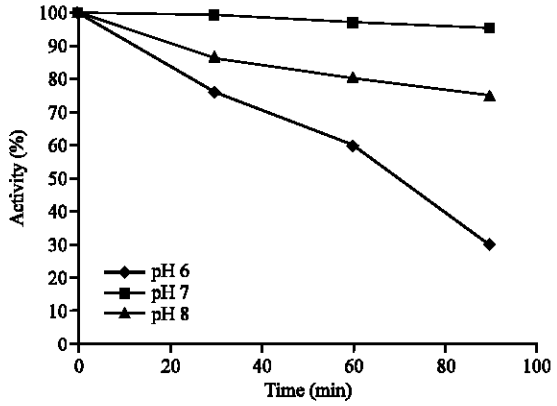

Fig. 3: Stability of partially purified protease at various pH. Enzyme activity was measured at time intervals of 30, 60 and 90 min. Each point represents the mean of three independent experiments. (100% activity corresponds to 30 U mL^{-1})

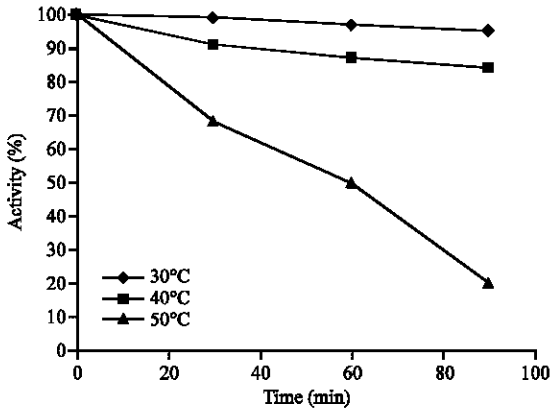

Fig. 4: Stability of partially purified protease at various temperatures. Enzyme activity was measured at time intervals of 30, 60 and 90 min. Each point represents the mean of three independent experiments. (100% activity corresponds to 30 U mL^{-1})

Stability study indicated that enzyme was able to retain 97 and 95% activity after exposing to pH 7.0 for 60 and 90 min, respectively. This is important since the lens cleansing formulation is mostly used in this pH range. Enzyme was able to retain about 75% activity at pH 8.0 after exposure for 90 min. However, there was a steep reduction in enzyme activity when exposed to pH 6.0 for time interval of 90 min (Fig. 3). This indicated that enzyme was most stable at pH 7. Johnvesly and Naik (2001) was reported the *Bacillus* sp. protease with stability in pH range of 6-11.

The enzyme was active in the temperature range of 20-60°C with maximum activity at 30°C, suggesting mesophilic nature of enzyme. However, 95 and 70% retained activities were observed at temperature 40 and 50°C, respectively. The enzyme was less active below 20°C and above 60°C (Fig. 2). Yossan *et al.* (2006) reported the optimum temperature of 50°C for *Bacillus megaterium* protease and retained the activity at 30-45°C with resulting relative activity of higher than 80%.

Enzyme was stable up to 30 min when exposed to 30 and 40°C and also retain 97 and 87% activity after 60 min. After 90 min of exposure 95% activity was detected at 30°C, however, there was slight decrease in activity at 40°C. The linear decreased stability was observed at 50°C with respect to different time exposure (Fig. 4). This indicated that enzyme was very stable at 30°C and moderately stable at 40°C. Cleansing of contact lenses is usually done at temperatures around 30°C, the since the enzyme is very stable at this temperature range it has much higher potential than the thermophilic protease.

In order to study the effectiveness of bacterial protease in removing proteinaceous deposits and debris from contact lenses, few lenses were coated with lysozyme followed by *Bacillus* sp. protease enzyme treatment. The spectroscopic analysis of the contact lens indicated that before coating the contact lenses with lysozyme the percent transmittance was 98% and in accordance with the earlier studies (Harris and Chamberlain, 1978), after deposition of protein, it reduced to 71% and after treatment with enzyme it was 97% (Table 1). Thus the increased transmittance indicated that enzyme has potential in removal of protein deposits from contact lens. Similarly, a post treatment transmittance of control using phosphate buffer was 71%, indicating no protein removal. Effects of treatment of lenses with

Table 1: Comparative data of transmission (%) and removal of protein deposits from contact lenses

	Transmittance (%)		
	30	60	90
Treatments	(min)		
Untreated lens	98	99	98
After coating with lysozyme	71	70	71
After treatment with partially purified protease	90	97	97

enzyme and phosphate buffer are statistically significant ($p<0.0001$). The optimal time for contact lenses cleansing was 60 min and later there was no protein removal. Greene *et al.* (1996) reported that the enzyme from marine bacterium degraded lysozyme, the major protein contaminant of contact lens and was effective in solution containing hydrogen peroxide.

Generally, contact lens cleansing is carried out with three types of cleaner solution as surfactant, oxidative and enzyme. Surfactants are safe and non-harmful to lenses but do not effectively remove the protein deposits. Oxidative cleaners are effective in removing non-protein deposits from contact lenses, however can have deleterious effect on lenses. Enzyme cleaners are safe to lenses and efficient in removing the main component of contact lens debris, namely proteins.

CONCLUSION

Protease isolated from *Bacillus* sp. 158 is active and stable in pH 7 and temperature 30°C, respectively. It has potential application in contact lens cleansing as a non-hazardous and bioalternative.

ACKNOWLEDGMENT

The study was carried out by financial support from Kelkar Education Trust, Mulund, India.

REFERENCES

Abidi, F., F. Limam and M.M. Nejib, 2008. Production of alkaline proteases by Botrytis cinerea using economic raw materials: Assay as biodetergent. Proc. Biochem., 43: 1202-1208.

Annapurna, R.A., N.K. Chandrababu, N. Samivelu, C. Rose and N.M. Rao, 1996. Eco- friendly enzymatic dehairing using extracellular protease from *Bacillus* species isolate. J. Am. Leather Chem. Assoc., 91: 115-119.

Anvar, A. and M. Saleemuddin, 1998. Alkaline proteases: A review. Biores. Technol., 64: 175-183.

Bruinsma, G.M., H.C. Van der Mei and H.J. Busscher, 2001. Bacterial adhesion to surface hydrophilic and hydrophobic contact lenses. Biomaterial, 22: 3217-3224.

Butrus, S.I. and S.A. Klotz, 1990. Contact lens surface deposits increase the adhesion of *Pseudomonas aeruginosa*. Curr. Eye Res., 9: 717-724.

George, S., V. Raju, M.V.R. Krishnan, T.V. Subramanian and K. Jayaraman, 1995. Production of protease by *Bacillus amyloliquefacian* in solid-state fermentation and its application in unhairing of hides and skins. Proc. Biochem., 30: 457-462.

Giongo, J.L., F.S. Lucas, F. Casarin, P. Heeb and A. Brandelli, 2007. Keratinolytic protease of *Bacillus* species isolated from the Amazon basin showing remarkable dehairing activity. World J. Microbiol. Biotechnol., 23: 375-382.

Greene, R.V., H.L. Griffin and M.A. Cotta, 1996. Utility of alkaline protease from marine shipworm bacterium in industrial cleansing applicat. Biotechnol. Lett., 18: 759-764.

Gupta, R., O.K. Beg and P. Lorenz, 2002. Bacterial alkaline proteases: Molecular approaches and industrial applications. Applied Microbiol. Biotechnol., 59: 15-32.

Haq, I., H. Mukhtar, S. Daudi, S. Ali and M.A. Qadeer, 2003. Production of proteases by a locally isolated mould culture under lab conditions. Biotechnology, 2: 30-36.

Harris, M.G. and M.D. Chamberlain, 1978. Light transmission of hydrogel contact lenses. Am. J. Optom. Physiol. Opt., 55: 93-96.

Harris, M.G., R.S. Chin, D.S. Lee, M.H. Tarot and C.E. Dobkins, 2000. Ultraviolet transmittance of the Vistakon disposable contact lenses. Contact Lens Anterior Eye, 23: 10-15.

Johnvesly, B. and G.R. Naik, 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. Proc. Biochem., 37: 139-144.

Joo, H.S. and C.S. Chang, 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: Optimization and some properties. Proc. Biochem., 40: 1263-1270.

Kumar, C.G. and H. Takagi, 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnol. Adv., 17: 561-594.

Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Nilegaonkar, S.S., V.P. Zambare, P.P. Kanekar, P.K. Dhakephalkar and S.S. Sarnaik, 2007. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. Biores. Technol., 98: 1238-1245.

Nunes, A.S. and M.L. Martins, 2001. Isolation, properties and kinetics of growth of a thermophilic *Bacillus*. Braz. J. Microbiol., 32: 271-275.

Sidler, W., B. Kumpf, B. Peterhans and H. Zehber, 1986. A neutral proteinase produced by *B. cereus* with high sequence homology to thermolysin: Production, isolation and characterization. Applied Microbiol. Biotechnol., 25: 18-24.

Singh, J., R.M. Vohra and D.K. Sahoo, 1999. Alkaline protease from a new obligate alkaophilic isolate of *Bacillus sphaericus*. Biotechnol. Lett., 21: 921-924.

Sneath, P.H.A., N.S. Mair, E.M. Sharpe and J.G. Holt, 2005. Bergey's Manual of Systematic Bacteriology. 2nd Edn., Williams and Wilkins, Baltimore, ISBN-10: 0387950400.

Tari, C., H. Genckal and F. Tokatli, 2006. Optimization of a growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp. L21. Proc. Biochem., 41: 659-665.

Yossan, S., A. Reungsang and M. Yasuda, 2006. Purification and characterization of alkaline protease from *B. megaterium* isolated from Thai fish sauce fermentation process. Sci. Asia, 32: 377-383.

Zambare, V.P., S.S. Nilegaonkar and P.P. Kanekar, 2007. Production of an alkaline protease and its application in dehairing of baffalo hide. World J. Microbiol. Biotechnol., 23: 1569-1574.